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Title	Characterization of H5N6 highly pathogenic avian influenza viruses isolated from wild and captive birds in the winter season of 2016-2017 in Northern Japan
Author(s)	Hiono, Takahiro; Okamatsu, Masatoshi; Matsuno, Keita et al.
Citation	Microbiology and immunology, 61(9), 387-397 https://doi.org/10.1111/1348-0421.12506
Issue Date	2017-08-16
Doc URL	https://hdl.handle.net/2115/71524
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Type	journal article
File Information	Revised Main text Hiono et al.pdf

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Original Article

Characterization of H5N6 highly pathogenic avian influenza viruses isolated from wild and captive birds in the winter season of 2016–2017 in northern Japan

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Running title: Characterization of H5N6 HPAIVs in Japan

ABSTRACT

On November 15, 2016, a suspected case of highly pathogenic avian influenza (HPAI) in a dead black swan was reported from a zoo in Akita prefecture, northern Japan, and an HPAI virus (HPAIV) belonging to the H5N6 subtype was isolated from specimens taken from the bird. After the initial report, 230 cases of HPAI caused by H5N6 viruses were reported from wild birds, captive birds, and domestic poultry farms throughout the country during the winter season. In the present study, we further characterized 66 H5N6 HPAIVs isolated from northern Japan. Phylogenetic analysis of the hemagglutinin gene showed that the H5N6 viruses isolated in northern Japan clustered into Group C of Clade 2.3.4.4 together with other isolates collected in Japan, Korea, and Taiwan during the winter season of 2016–2017. The antigenicity of the Japanese H5N6 isolate differed slightly from that of HPAIVs isolated previously in Japan and China. The virus exhibited high pathogenicity and a high replication capacity in chickens, whereas virus growth was slightly lower in ducks compared with an H5N8 HPAIV isolate collected in Japan in 2014. Comprehensive analyses of Japanese isolates, including those from central, western, and southern Japan, as well as rapid publication of this information are essential to facilitate greater control of HPAIVs.

Key Words: H5N6, highly pathogenic avian influenza virus, wild bird

INTRODUCTION

During the winter season of 2014–2015, highly pathogenic avian influenza viruses (HPAIVs) spread throughout the world (1), where 14 countries and regions in the Eurasian and American continents were affected by infections with HPAIVs. These viruses possess hemagglutinin (HA) genes that belong to genetic Clade 2.3.4.4 as well as neuraminidase (NA) genes originating from viruses maintained in local poultry or wild birds. At present, these viruses referred to as H5Nx viruses, which have various NA subtypes are further divided into four genetically distinct groups: Groups icA, B, C, and D (2, 3). In the season, viruses belonging Group icA widely disseminated, whilst viruses of the other groups were limited in East and Southeast Asia (3, 4). During the winter season of 2016–2017, H5Nx viruses again spread throughout three continents: Eurasia, North America, and Africa (1). It was reported that H5N8 viruses belonging Clade 2.3.4.4 Group B were isolated in Europe, Iran, and Egypt (5-8), while H5N6 viruses belonging Clade 2.3.4.4 Group C were isolated in East Asia (9-10). Detailed analyses of the isolates are still on going. For the better control of HPAIVs coursed by H5Nx viruses, rapid publication of the information on outbreaks and virus isolates are essential.

On November 15, 2016, a suspected case of HPAI in a dead black swan was reported from a zoo in the Akita prefecture, northern Japan, and an HPAIV belonging to the H5N6 subtype was isolated from specimens taken from the bird. The HA gene of the virus was classified into Group C of clade 2.3.4.4 and it was closely related to those in the viruses isolated in China (9). After the initial report, 178 cases in wild birds, 40 cases in captive birds, including the initial case, and 12 outbreaks in domestic poultry farms were reported with H5N6 HPAIVs throughout Japan by March 24, 2017, when the final incident was documented at a poultry farm in Chiba prefecture (Fig. S1) (11). After that, Japan recovered HPAI free status. In the present study, we further characterized the H5N6 HPAIVs isolated in Japan, especially 66 strains isolated in northern Japan.

MATERIALS AND METHODS

Viruses

An HPAIV, A/chicken/Kuamoto/1-7/2014 (H5N8) was kindly provided by Dr Takehiko Saito, National institute of Animal Health (NIAH), Japan. A/duck/Japan/AQ-HE72/2015 (H5N6) was isolated from raw duck meat, which was illegally imported into Japan by international flight passengers from China (Shibata et al., submitted). A/duck/Vietnam/HU1-1151/2014 (H5N6) was isolated from a duck acquired from a live bird market in Vietnam (12). A/chicken/Hokkaido/002/2016 (H5N6) was identified in our laboratory in a hemagglutination-positive sample of allantoic fluid, which was kindly provided by the Hokkaido prefecture, Japan. The sample was originally obtained from a domestic chicken farm in the Hokkaido Prefecture, which was later confirmed to be affected with HPAI by NIAH (on December 16, 2016). The viruses were propagated in 10-day-old embryonated chicken eggs at 35 °C for 36–48 h and the infectious allantoic fluids were used as virus stocks.

Isolation and identification of viruses from wild birds

Virus isolation was performed using tracheal and cloacal swabs. Swabs were collected by local officers and shipped to the Laboratory of Microbiology, Faculty of Veterinary Medicine, Hokkaido University. The swabs were then filtered through a 0.45-μm filter (DISMIC-25SS, ADVANTEC, Tokyo, Japan) and mixed with virus transport medium containing minimum essential medium (Nissui Pharmaceutical, Tokyo, Japan), 10,000 U/m*l* penicillin G (Meiji Seika Pharma, Tokyo, Japan), 10 mg/m*l* streptomycin (Meiji Seika Pharma), 0.3 mg/m*l* gentamicin (MSD Corporation, Tokyo, Japan), 250 U/m*l* nystatin (Sigma Aldrich, St Louis, MO, USA), and 0.5% bovine serum albumin fraction V (Roche, Basel, Switzerland) at a ratio of 1:1. Samples were inoculated into the allantoic cavities of nine- to 11-day-old embryonated chicken eggs, and allantoic fluid showing hemagglutination was collected as virus-containing solution. The HA and NA subtypes of the

92 influenza virus isolates were identified using hemagglutination inhibition (HI) and NA inhibition
93 tests, respectively, according to a standard protocol (12). For some viruses, the HA subtype was
94 identified with an immunochromatographic kit (New Linjudge Flu A/H5; TAUNS Laboratories, Inc.
95 Shizuoka, Japan; Nguyen et al., submitted). In total, 66 strains were isolated in the present study
96 (Table 1).
97
98 **Gene sequencing and phylogenetic analysis**
99 Viral RNA was extracted from the allantoic fluids of embryonated chicken eggs using TRIzol
100 LS Reagent (Thermo Fisher Scientific, Waltham, MA, USA), and reverse transcribed with the Uni12
101 primer (14) and SuperScript IV Reverse Transcriptase (Thermo Fisher Scientific). The partial
102 sequence of the HA gene segment was amplified by polymerase chain reaction with the gene-specific
103 primer sets HA-155F: ACACATGCYCARGACATACT and HA-1201R:
104 GTGTTCATTTTGTYAATGAT. The nucleotide sequences of the amplified fragments were
105 determined using BigDye terminator v3.1 (Thermo Fisher Scientific) and an auto-sequencer (3500
106 Genetic Analyzer; Thermo Fisher Scientific). The whole genome sequences were determined for the
107 26 representative examples of the 66 strains using the deep sequencing method. Briefly, MiSeq
108 libraries were prepared using an NEBNext Ultra RNA Library Prep Kit for Illumina (New England
109 Biolabs, Ipswich, MA, USA) and sequenced on the MiSeq system with a MiSeq reagent kit v3
110 (Illumina, San Diego, CA, USA). Reads were assembled *de novo* using CLC Genomic Workbench
111 (CLC bio, Aarhus, Denmark). The nucleotide sequences were phylogenetically analyzed based on
112 the maximum-likelihood method according to the Tamura-Nei model and bootstrap analysis (n =
113 1,000) using MEGA 7.0 software (15) with the default parameters. Sequence data for genes were
114 compared with reference sequences obtained from GenBank/EMBL/DDBJ and GISAID.
115
116 **Antigenic analysis**

The antigenic properties of the representative H5 viruses were assessed using hyperimmunized chicken antisera against A/mallard/Hokkaido/24/2009 (H5N1), A/chicken/Kumamoto/1-7/2014 (H5N8), and A/black swan/Akita/1/2016 (H5N6) with the HI test according to a standard protocol (12). HI titers were expressed as the reciprocals of the highest serum dilutions that exhibited complete HI. A/whooper swan/Fukushima/1/2016 (H5N6), A/whooper swan/Iwate/5/2016 (H5N6), and A/pintail/Hokkaido/X8/2016 (H5N6) were selected as representative strains based on relatively low-genetic similarity in the HA genes with A/black swan/Akita/1/2016 (H5N6) among the isolates in the present study (99.2, 99.2, and 99.1%, respectively). A/chicken/Hokkaido/002/2016 (H5N6), which is a poultry isolate was also used in the antigenic analysis.

Animal experiments

Seven-week-old chickens (*Gallus gallus*, Julia) were obtained from Hokkai Starchick, Hokkaido, Japan. Four chickens were inoculated intranasally with 100 μl of virus solution containing a $10^{6.0}$ 50% infectious dose for 10-day-old embryonated chicken eggs (EID_{50}) with A/black swan/Akita/1/2016 (H5N6). At 3 days post-inoculation (dpi), swab (oral and cloacal), blood, and tissue (brain, trachea, lung, kidney, and colon) samples were collected from the dead birds. Four-week-old domestic ducks (*Anas platyrhynchos* var. *domesticus*, Cherry Valley) were obtained from Takikawa Shinseien, Hokkaido, Japan. Eight ducks were also inoculated intranasally with 100 μl of virus solution containing $10^{6.0}$ EID_{50} of A/black swan/Akita/1/2016 (H5N6). At 3 dpi, four individuals were euthanized and swab, blood, and tissue samples were collected. The tissue samples were homogenized using a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan) in order to prepare a 10% suspension in virus transport medium. The infectivity titers for swab, blood, and tissue samples were calculated based on the 50% tissue culture infectious dose ($TCID_{50}$) using Madin–Darby canine kidney (MDCK) cells. The other four birds were kept for 14 days to observe their clinical signs. To calculate the 50% chicken lethal dose (CLD_{50}) for A/black swan/Akita/1/2016 (H5N6), $10^{7.0}$, $10^{6.0}$,

142 $10^{5.0}$, $10^{4.0}$, $10^{3.0}$, or $10^{2.0}$ EID_{50} of the virus was inoculated intranasally into each of four
143 seven-week-old chickens. The chickens were kept for 14 days to observe their survival rates. All of
144 the infected animals were kept in self-contained isolator units (Tokiwa Kagaku, Tokyo, Japan) in the
145 BSL3 biosafety facility of the Faculty of Veterinary Medicine, Hokkaido University, Japan.
146
147 **Ethics statements**
148 All of the animal experiments were authorized by the Institutional Animal Care and Use
149 Committee of the Faculty of Veterinary Medicine, Hokkaido University (approval number: 13-050).
150 All experiments were performed according to the guidelines of the committee. The BSL3 biosafety
151 facility of the Faculty of Veterinary Medicine, Hokkaido University is permitted to store and use
152 HPAIVs by Ministry of Agriculture, Forestry, and Fisheries based on Act on Domestic Animal
153 Infectious Diseases Control (permission number: 19).
154
155 **RESULTS**
156 **Isolation and identification of H5N6 HPAIVs from wild and captive birds**
157 By March 24, 2017, 66 HPAI cases in wild and captive birds from northern Japan were tested in
158 our laboratory. The host species and geographic locations of these cases are listed in Table 1. All of
159 the isolates were identified as belonging to the H5N6 subtype. Multiple basic amino acids
160 (RERRRKR/GLF) were found at the cleavage site of the deduced amino acid sequence for HA in all
161 66 isolates, thereby suggesting high pathogenicity in chickens.
162
163 **Phylogenetic analysis of the H5N6 isolates**
164 The partial sequences (position 147 to 1194, where the adenine residue of start codon was
165 counted as position 1 and the sequence of A/black swan/Akita/1/2016 (H5N6) was used as a
166 reference sequence) of the HA genes of all 66 isolates were phylogenetically analyzed with reference

sequences, including recent H5N6 isolates from Korea and Taiwan (Fig. 1). The HA genes of all 66 isolates were phylogenetically closely related to the initial Japanese isolate, A/black swan/Akita/1/2017 (H5N6) (9). The HA gene of a poultry isolate, A/chicken/Hokkaido/002/2016 (H5N6) (GISAID accession numbers: EPI962342-49), was also closely related to those of the viruses isolated in the present study. Moreover, all the H5N6 viruses isolated in Japan, Korea, and Taiwan during the winter season of 2016–2017 formed one cluster with a high bootstrap value (99%) in Group C of Clade 2.3.4.4. The sequences of the HA genes from Japanese, Korean, and Taiwanese H5N6 isolates were highly conserved. The NA genes of these isolates also formed a single cluster together with the H5N6 HPAIVs isolated from China in Group C (Fig. S2a). The other six internal genes (except the PA genes) of the Japanese, Korean, and Taiwanese H5N6 viruses were closely related in the corresponding trees (Fig. S2b–f). However, the PA genes of the Japanese, Korean, and Taiwanese H5N6 viruses were divided into three groups according to the phylogenetic tree (Fig. 2). Most of the viruses isolated in the present study formed a single cluster with A/black swan/Akita/1/2016 (H5N6) and with Chinese H5N6 HPAIVs (FL/GD-like). The PA gene of A/white-fronted goose/Miyagi/1/2016 (H5N6) clustered with those of A/mallard/Jianxi/8264/2004 (H6N2) and A/chicken/Shantou/212/2000 (H9N2), and with a western Japanese isolate, A/teal/Tottori/2/2016 (H5N6), as well as with Korean and Taiwanese strains (Ck/ST/212-like). As reported by Lee et al. (10), the PA genes of several Korean isolates were phylogenetically related to those of Eurasian nonpathogenic avian influenza viruses isolated from migratory birds.

Antigenic analysis of H5N6 HPAIV isolates

H5 HPAIVs with HA genes belonging to Clade 2.3.4.4 were analyzed antigenically using hyperimmunized chicken antisera to A/mallard/Hokkaido/24/2009 (H5N1), A/chicken/Kumamoto/1-7/2014 (H5N8), and A/black swan/Akita/1/2016 (H5N6) (Table 2). A/mallard/Hokkaido/24/2009 (H5N1) is a non-pathogenic avian influenza virus isolated from a fecal

192 sample from migratory ducks and the antigenicity of this strain is similar to those of other H5 viruses
193 isolated from migratory ducks (16). A/chicken/Kumamoto/1-7/2014 (H5N8) was isolated from a
194 chicken in 2014 at Kumamoto prefecture, Japan, and the HA gene of this strain belongs to Group icA
195 in clade 2.3.4.4 (17). A/duck/Japan/AQ-HE72/2015 (H5N6) was isolated from raw chicken meat,
196 which was illegally imported into Japan by international flight passengers from China, and the HA
197 gene of this strain belongs to Group C in Clade 2.3.4.4 (Shibata et al., submitted).
198 A/duck/Vietnam/HU1-1151/2014 (H5N6) was isolated from a duck acquired from a live bird market
199 in Vietnam and the HA gene of this strain belongs to Group D in clade 2.3.4.4 (12). As expected, the
200 reactivity of all four H5Nx HPAIVs with the antiserum to A/mallard/Hokkaido/24/2009 (H5N1) was
201 quite low. As reported by Okamatsu et al. (9), the HI titer for A/black swan/Akita/1/2016 (H5N6)
202 was eight times lower with antiserum to A/chicken/Kumamoto/1-7/2014 (H5N8) compared with the
203 homologous titer. By contrast, the HI titer for A/chicken/Kumamoto/1-7/2014 (H5N8) was four
204 times lower with antiserum to A/black swan/Akita/1/2016 (H5N6) compared with the homologous
205 titer. The HI titer for A/duck/Japan/AQ-HE72/2015 (H5N6) with antiserum to
206 A/chicken/Kumamoto/1-7/2014 (H5N8) was comparable to the homologous titer, although both
207 A/duck/Japan/AQ-HE72/2015 (H5N6) and A/black swan/Akita/1/2016 (H5N6) belong to Group C
208 of Clade 2.3.4.4. Moreover, other viruses isolated in the winter season of 2016-2017 in Northern
209 Japan showed similar reactive patterns to each of antisera as A/black swan/Akita/1/2016 (H5N6) did.
210 Thus, the antigenicity of H5N6 viruses belonging to Group C in Clade 2.3.4.4 differed slightly
211 within the same group.
212
213 **Pathogenicity of H5N6 viruses in chickens and ducks**
214 To assess the pathogenicity of H5N6 HPAIVs isolated in Japan in chickens, an index strain
215 A/black swan/Akita/1/2016 (H5N6) was inoculated intranasally into seven-week-old chickens. All
216 the chickens inoculated with the virus died at 3 dpi. The virus was recovered from swab, blood, and

tissue samples collected from the dead birds, with high titers (Table 3). The CLD_{50} value calculated for A/black swan/Akita/1/2016 (H5N6) was $10^{4.3}$ EID_{50}. To assess the pathogenicity in ducks of H5N6 HPAIVs isolated in Japan, A/black swan/Akita/1/2016 (H5N6) was also inoculated intranasally into 4-week-old ducks. Virus growth by A/black swan/Akita/1/2016 (H5N6) was lower in ducks than chickens. All of the inoculated ducks survived throughout the 14-day observation period and shed virus, but they exhibited no clinical signs.

DISCUSSION

After the outbreak of H5Nx HPAI during the winter season of 2014–2015, great efforts were made to identify the route of virus transmission via migratory birds (2, 4). In Japan, at least three independent virus introductions were suggested based on phylogenetic analyses (17). However, a comprehensive understanding of the role of migratory birds in the spread of HPAIVs has not yet been achieved. In the present study, phylogenetic analysis of the HA gene segments showed that the H5N6 viruses isolated in northern Japan were phylogenetically closely related, with common ancestors (Fig. 1). However, the PA genes of these viruses clustered into two distinct groups according to the phylogenetic tree (Fig. 2), thereby supporting the hypothesis of multiple events of virus introduction. Lee et al. (10) reported that Korean H5N6 viruses could be classified into three genotypes based on the origin of the PA gene segment. In addition, the majority of the Japanese H5N6 viruses possessed PA genes that shared high similarity with Chinese H5N6 HPAIVs, thereby indicating that the origin of the PA gene in the Japanese strains was highly biased compared with that in the Korean strains. The bias may suggest that the H5N6 HPAIVs were introduced independently into Japan and Korea from nesting areas for wild ducks or swans in their northern territory, and the viruses were not carried via the Korean peninsula to Japan via migrating birds. On the other hand, it is possible that only a small portion of the viruses circulating in Korea was introduced to Japan via Korean peninsula along with migratory birds. Thus, in order to clarify the

242 transmission route for the H5N6 HPAIVs to Japan, comprehensive analyses of Japanese isolates,
243 including those from central, western, and southern Japan, should be required.

244 Several areas of Japan were highly affected where 10 or more cases were documented during
245 the season. In particular, two lakes in the Niigata and Ibaraki prefectures (latitude: 37.8384°N,
246 longitude: 139.2373°E and latitude: 36.3660°N, longitude: 140.4716°E) are well-known as
247 over-wintering areas for swans (Table 1, Fig. S1). The viral isolates from these areas shared high
248 genetic similarity (Fig. 1). However, accumulated common mutation(s) in the HA genes was not
249 observed in the Japanese isolates, thereby implying that virus transmission among wild birds
250 occurred only in a limited area and population, and that viruses died out almost completely after a
251 certain period. Nevertheless, HPAIVs were isolated in January and February when long-distance
252 flights by birds are not usually observed, and thus the diseased birds were considered to have
253 overwintered in Japan (Table 1). This suggests that H5N6 viruses might have been maintained in
254 Japan for a certain period. The introduction of the viruses and their spread throughout Japan are not
255 clearly understood at present. Thus, continued monitoring of wild bird movements and the viruses
256 that circulate among them are essential for facilitating the control of H5Nx HPAIVs in Japan.

257 Antigenic analysis of Clade 2.3.4.4 viruses showed that the antigenicity of H5N6 viruses
258 belonging to Group C in Clade 2.3.4.4 differed slightly within this group (Table 2). Compared with
259 the mature HA amino acid sequence of A/duck/Japan/AQ-HE72/2015 (H5N6), only nine residues
260 differed in that of A/black swan/Akita/1/2016 (H5N6) (data not shown), including a deletion of
261 leucine at 134 and a mutation from arginine to glutamine at 227, which are commonly found in
262 Japanese and Korean H5N6 HPAIVs according to Okamatsu et al. (9). The roles of these mutations
263 in viral antigenicity and the function of HA should be clarified. Furthermore, continued monitoring
264 of antigenicity may show whether these antigenically drifted viruses can overwhelm the currently
265 present viruses or not.

Experimental infection of chickens demonstrated the high pathogenicity of A/black swan/Akita/1/2016 (H5N6). The CLD_{50} value calculated for A/black swan/Akita/1/2016 (H5N6), i.e., $10^{4.3}$ EID_{50}, was lower than that for a Clade 2.3.4.4icA virus, A/chicken/Kumamoto/1-7/2014 (H5N6) (1 CLD_{50} = $10^{5.8}$ EID_{50}) (18). Previous studies suggested that viruses belonging to Clade 2.3.4.4icA are less pathogenic in chickens compared with previously known HPAIVs (19-21), where this was explained as a consequence of adaptation to waterfowl. The CLD_{50} value calculated for A/black swan/Akita/1/2016 (H5N6) was comparable to those obtained for other HPAIVs (22). In contrast to the virus growth in chickens, virus growth by A/black swan/Akita/1/2016 (H5N6) was slightly lower in ducks compared with that by A/chicken/Kumamoto/1-7/2014 (H5N8), which was studied by Kanehira et al. and Hiono et al. (19, 20). The molecular mechanisms that determine the host preferences of HPAIVs have been studied previously, especially the preferences for chickens and ducks. A previous study indicated that recent H5Nx viruses exhibited alterations in their receptor binding specificity compared with previous HPAIVs (23), where these viruses preferred fucosylated α2,3 sialosides in addition to typical non-fucosylated α2,3 sialosides. Fucosylation of α2,3 sialosides is thought to be a molecular determinant of host preferences by avian influenza viruses in ducks and chickens, where chicken viruses prefer fucosylated α2,3 sialosides and duck viruses prefer non-fucosylated α2,3 sialosides (24). All the viruses isolated in the present study possessed a glutamine at position 222 of the HA, which is a key amino acid residue for the recognition of fucosylated α2,3 sialosides. On the other hand, these viruses possessed glutamine at another key position, 227, where viruses of clade 2.3.4.4icA, represented by A/chicken/Kumamoto/1-7/2014 (H5N8), possessed arginine. The amino acid substitution may alter their pathogenicity to ducks and chickens. However, multiple viral factors are considered to be related to the pathogenesis of HPAIVs in ducks (25). Thus, obtaining a comprehensive understanding of the underlying mechanisms is essential for preventing viruses spreading from chickens to wild migratory ducks.

H5Nx viruses are currently a global concern. The introduction of H5Nx viruses into wild bird populations is one of the main reasons why these viruses have been disseminated so widely. These viruses are now spreading throughout the world via migrating wild waterfowl. Thus, the rapid publication of epidemiological information about outbreaks as well as genetic information regarding the causative agents are essential for facilitating greater control of the H5Nx viruses. Moreover, HPAIVs must be urgently eradicated from poultry in endemic countries in order to prevent virus spill-over into wild bird populations.

ACKNOWLEDGMENTS

We deeply appreciate the kind cooperation of the Ministry of Environment and Ministry of Agriculture, Forestry, and Fisheries, Government of Japan. We also thank Ms M. Endo, Ms K. Oka, and Ms A. Ohnuma of the Hokkaido University for their technical support. We thank the Livestock Hygiene Service Office, Hokkaido Prefecture for providing the hemagglutination-positive sample from which A/chicken/Hokkaido/002/2016 (H5N6) was isolated. We thank Dr T. Saito of the Influenza and Prion Diseases Research Center, NIAH, Japan for kindly providing A/chicken/Kumamoto/1-7/2014 (H5N8). The present study was supported in part by the J-GRID; the Japan Initiative for Global Research Network on Infectious Diseases and Japan Science and Technology Agency Basic Research Programs.

DISCLOSURE

The authors have no conflicts of interest to declare.

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403 **Figure Legends**

404 **Fig. 1 Phylogenetic tree obtained based on the HA genes of H5N6 HPAIVs isolated in Japan.**
405 The HA genes of 66 HPAIVs isolated in the present study as well as those of reference strains were
406 analyzed using the maximum-likelihood method with MEGA 7 software (15). Nucleotide sequences
407 from position 147 to 1194, where the adenine residue of start codon was counted as position 1 and
408 the sequence of A/black swan/Akita/1/2016 (H5N6) was used as a reference sequence, were
409 incorporated in the dataset. Horizontal distances are proportional to the minimum number of
410 nucleotide differences required with respect to the nodes and sequences. Numbers at the nodes
411 indicate the probability of the confidence levels according to bootstrap analysis based on 1,000
412 replicates. A poultry isolate, A/chicken/Hokkaido/002/2016 (H5N6) was indicated with a circle filled
413 with white. Circles filled with black indicate other H5N6 HPAIVs isolated in Japan. Viruses isolated
414 in the present study are underlined. The viruses were divided into four groups (icA, B, C, and D)
415 based on Lee et al. (3).

416

417 **Fig. 2 Phylogenetic tree obtained based on the PA genes of H5N6 HPAIVs isolated in Japan.**
418 The PA genes of 26 representative HPAIVs isolated in the present study as well as those of reference
419 strains were analyzed using the maximum-likelihood method with MEGA 7 software (15).
420 Nucleotide sequences from position 30 to 2147, where the adenine residue of start codon was
421 counted as position 1 and the sequence of A/black swan/Akita/1/2016 (H5N6) was used as a
422 reference sequence, were incorporated in the dataset. Horizontal distances are proportional to the
423 minimum number of nucleotide differences required with respect to the nodes and sequences.
424 Numbers at the nodes indicate the probability of the confidence levels according to bootstrap
425 analysis based on 1,000 replicates. A poultry isolate, A/chicken/Hokkaido/002/2016 (H5N6) was
426 indicated with a circle filled with white. Circles filled with black indicate other H5N6 HPAIVs
427 isolated in Japan. Viruses isolated in the present study are underlined. FL/GD:

A/feline/Guangdong/1/2015 (H5N6); NPAIV: non-pathogenic avian influenza virus; Ck/ST/212: A/chicken/Shantou/212/2000 (H9N2).

Supporting Information

Fig. S1 H5N6 HPAIV infections in wild birds, captive birds, and domestic poultry in 2016-2017 winter season in Japan. HPAIVs belonging to the H5N6 subtype was isolated from 178 wild birds (denoted by red circles), 40 captive birds (denoted by green circles), and 12 farms of domestic poultry (denoted by blue circles) during the winter season of 2016-2017. The occurrences at different geographical location were indicated by circles, and the subsequent cases at the same place were omitted. The specimens used in the present study were collected in the northern area of Japan segregated by dashed line.

Fig. S2 Phylogenetic tree obtained based on the NA, PB2, PB1, NP, M, and NS genes of H5N6 HPAIVs isolated in Japan. The NA (a), PB2 (b), PB1(c), NP(d), M(e), and NS(f) genes of 26 representative HPAIVs isolated in the present study as well as those of reference strains were analyzed using the maximum-likelihood method with MEGA 7 software (15). Horizontal distances are proportional to the minimum number of nucleotide differences required with respect to the nodes and sequences. Numbers at the nodes indicate the probability of the confidence levels according to bootstrap analysis based on 1,000 replicates. A poultry isolate, A/chicken/Hokkaido/002/2016 (H5N6) was indicated with a circle filled with white. Circles filled with black indicate other H5N6 HPAIVs isolated in Japan. Viruses isolated in the present study are underlined.

List of the abbreviations

Abbreviations	Definitions
CLD_{50}	50% chicken lethal dose
dpi	days post-inoculation
EID_{50}	50% infectious dose for 10-day-old embryonated chicken eggs
HA	hemagglutinin
HI	hemagglutination inhibition
HPAI	highly pathogenic avian influenza
HPAIV	highly pathogenic avian influenza virus
MDCK	Madin–Darby canine kidney
NA	neuraminidase
NIAH	National Institute of Animal Health
$TCID_{50}$	50% tissue culture infectious dose